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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/12, 5/10, C07K 14/47, 16/18, 16/42, C12Q 1/68, G01N 33/574, A61K 38/17, 31/70, 39/395		A2	(11) International Publication Number: WO 99/37775
			(43) International Publication Date: 29 July 1999 (29.07.99)
(21) International Application Number: PCT/US99/01556		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW; ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 25 January 1999 (25.01.99)		Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(30) Priority Data: 60/072,526 26 January 1998 (26.01.98) US			
(71) Applicant: GENQUEST, INC. [US/US]; The Audubon Technology Center, 4th floor, 3960 Broadway, New York, NY 10032 (US).			
(72) Inventors: HUANG, Fei; 8404 58th Avenue, Elmhurst, NY 11373 (US). ADELMAN, Jennifer, R.; 646 74th Street, Brooklyn, NY 11209 (US). FISHER, Paul, B.; 15 Gordon Place, Scarsdale, NY 10583-1938 (US). GOLDSTEIN, Neil, I.; 26 Kendal Avenue, Maplewood, NJ 07040 (US).			
(74) Agents: MAKI, David, J. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).			

(54) Title: COMPOSITIONS AND METHODS FOR DETECTING AND TREATING BREAST CANCER

(57) Abstract

Compositions and methods for the detection and therapy of breast cancer are disclosed. The compounds provided include tumor-associated proteins, and portions and variants thereof, as well as polynucleotides that encode such polypeptides. Vaccines and pharmaceutical compositions comprising such compounds are also provided and may be used, for example, for cancer prevention and treatment. The polypeptides may also be used for the production of antibodies, which are useful for diagnosing and monitoring cancer progression in a patient.

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COMPOSITIONS AND METHODS FOR DETECTING AND TREATING BREAST CANCER

TECHNICAL FIELD

The present invention relates generally to the detection and therapy of cancer, such as breast cancer. The invention is more specifically related to tumor-associated polypeptides and nucleotide sequences, and variants thereof, which may be used in vaccines and pharmaceutical compositions for preventing and treating cancer. The polypeptides may also be used for the production of compounds, such as antibodies, useful for diagnosing and monitoring the progression of a cancer in a patient.

BACKGROUND OF THE INVENTION

Cancer is a significant health problem in the United States and throughout the world. Although advances have been made in detection and treatment of cancer, no vaccine or other universally successful method for cancer prevention or treatment is currently available. Management of the disease currently relies on a combination of early diagnosis and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality continues to be observed in many cancer patients.

Breast cancer is a significant health problem for women in the United States and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the second leading cause of cancer-related deaths in women, affecting more than 180,000 women in the United States each year. For women in North America, the life-time odds of getting breast cancer are now one in eight.

No vaccine or other universally successful method for the prevention or treatment of breast cancer is currently available. Management of the disease currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular breast cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. See, e.g., Porter-Jordan and Lippman, *Breast Cancer* 8:73-100 (1994). However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality observed in breast cancer patients indicates that improvements are needed in the treatment, diagnosis and prevention of the disease.

Accordingly, there is a need in the art for improved methods for therapy and diagnosis of cancer, such as breast cancer. The present invention fulfills these needs and further provides other related advantages.

15 SUMMARY OF THE INVENTION

Briefly stated, this invention provides compositions and methods for cancer diagnosis and therapy. Within certain aspects, the present invention provides isolated polypeptides comprising an immunologically active portion of a tumor-associated protein or a variant thereof. In one such aspect, the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:1 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:1. Within another such aspect, the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:2 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:2.

Within further aspects, the present invention provides isolated polynucleotides comprising at least 10 nucleotides that encode a portion of a tumor-associated protein or a variant thereof. In certain such aspects, the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:1 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:1. Within other such aspects, the tumor-associated protein comprises an

amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:2 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:2.

Expression vectors comprising any of the above polynucleotides, and host cells transformed or transfected with such expression vectors, are also provided herein.

5 Within other aspects, monoclonal antibodies, or antigen-binding fragments thereof, that specifically bind to a polypeptide as provided above are provided.

The present invention further provides, within other aspects, T cells that specifically react with a polypeptide as described above, and antigen-presenting cells

10 that express such a polypeptide.

Within further aspects, the present invention provides pharmaceutical compositions, comprising a polypeptide, polynucleotide, antibody, T cell or antigen-presenting cell as described above, in combination with a physiologically acceptable carrier.

15 The present invention further provides, within other aspects, vaccines comprising a polypeptide, polynucleotide or antigen-presenting cell as described above and a nonspecific immune response enhancer.

Within further aspects, the present invention provides methods for inhibiting the development of breast cancer in a patient, comprising administering to a 20 patient a pharmaceutical composition or vaccine as described above.

The present invention further provides, within other aspects, methods for determining the presence or absence of breast cancer in a patient comprising assaying a biological sample obtained from a patient for the presence of a polypeptide or polynucleotide as described above, and therefrom determining the presence or absence 25 of breast cancer in the patient.

Within related aspects, the present invention provides methods for monitoring the progression of breast cancer in a patient, comprising: (a) detecting, in a biological sample obtained from a patient, an amount of a polypeptide or RNA molecule as provided above at a first point in time; (b) repeating step (a) at a subsequent

point in time; and (c) comparing the amounts of polypeptide detected in steps (a) and (b), and therefrom monitoring the progression of breast cancer in the patient.

The present invention further provides methods for preparing a polypeptide as described above, comprising the steps of: (a) culturing a host cell as described above under conditions suitable for the expression of the polypeptide; and (b) recovering the polypeptide from the host cell culture.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the partial sequence of a breast tumor-associated polynucleotide referred to herein as B7 (SEQ ID NO:1).

Figure 2 depicts the partial sequence of a breast tumor-associated polynucleotide referred to herein as B11 (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for cancer diagnosis, monitoring and therapy. The compositions described herein may include one or more polypeptides, nucleic acid sequences and/or antibodies. Polypeptides of the present invention generally comprise at least a portion of a tumor-associated protein, or a variant thereof. Nucleic acid sequences of the subject invention generally comprise a DNA or RNA sequence that encodes such a polypeptide, or that is complementary to such a coding sequence. Antibodies are generally immune system proteins, or antigen-binding fragments thereof, that are capable of binding to a portion of a polypeptide as described above. Alternatively, or in addition, a composition may comprise antigen-presenting cells (APC; e.g., dendritic cells) that express a polypeptide as provided herein and/or immune system cells (e.g., T cells, including CD4⁺ and/or CD8⁺) specific for such a polypeptide.

The present invention is based, in part, on the identification of breast tumor-associated cDNA and protein sequences. A tumor-associated cDNA molecule comprises a nucleotide sequence that corresponds to the sequence of a tumor-associated mRNA (and/or a complementary sequence). Such cDNA molecules may be prepared from tumor RNA using standard techniques, such as reverse transcription. As used herein, a "tumor-associated" mRNA is a mRNA that is expressed at a greater level in a human tumor tissue than in the corresponding normal tissue (i.e., the level of RNA is at least 2-fold higher in tumor tissue). Similarly, a tumor-associated protein or polypeptide comprises a sequence encoded by a tumor-associated mRNA, where the 5 protein or polypeptide is present at a greater level in a human tumor tissue than in the corresponding normal tissue (i.e., the level of protein is at least 2-fold higher in tumor tissue). In particular, the tumor associated sequences provided herein are expressed at greater levels in breast tumors than in normal breast tissue.

15 TUMOR-ASSOCIATED POLYNUCLEOTIDES

Any polynucleotide that encodes a tumor-associated polypeptide, or a portion or variant thereof as described herein, is encompassed by the present invention. Such polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding 20 or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Tumor-associated polynucleotides may be prepared using any of a variety of techniques. For example, such a polynucleotide may be amplified from 25 human genomic DNA, or from tumor cDNA, via polymerase chain reaction (PCR). For this approach, sequence-specific primers may be designed based on the sequences provided herein, and may be purchased or synthesized. An amplified portion may then be used to isolate a full length gene from a human genomic DNA library or from a tumor cDNA library, using well known techniques, as described below. Alternatively, a 30 full length gene can be constructed from multiple PCR fragments.

cDNA molecules encoding a native tumor-associated protein, or a portion thereof, may also be prepared by screening a cDNA library prepared from mRNA of a tumor tissue, such as a breast tumor tissue. Such libraries may be commercially available, or may be prepared using standard techniques (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989, and references cited therein). A library may be a cDNA expression library and may, but need not, be subtracted using well known subtractive hybridization techniques.

There are many types of screens that may be employed, including any of a variety of standard hybridization methods. For initial screens, conventional subtractive hybridization techniques may be used. Alternatively, cDNA molecules encoding tumor-associated proteins may be identified using techniques referred to herein as Tumor Rapid Expression Cloning System (T-RexCS) and Tumor Subtraction Rapid Expression Cloning System (TS-RexCS). Briefly, such methods comprise the steps of (1) transfecting tumor-derived cDNA expression libraries (prepared in a vector containing a gene conferring selectable antibiotic resistance from cell lines or from primary or metastatic cancer tissues) into non-tumorigenic cells; (2) injecting pooled colonies into immunocompromised animals (*i.e.*, non-human animals whose ability to mount an immune response is detectably impaired) such as nude, mice SCID mice or XID mice; (3) excising any tumors that are formed and explanting the tumors in tissue culture dishes containing medium supplemented with fetal bovine serum and the selecting antibiotic; (4) isolating cells that grow from the explants (*e.g.*, with trypsin); and (5) identifying oncogenes and tumor associated antigens from such cells by any suitable immunological or gene cloning technique, such as SEM, phage rescue, PCR, differential display or subtraction hybridization. TS-RexCS is identical to T-RexCS except that the cDNA library is first subtracted with a normal cDNA library.

A tumor-associated cDNA molecule may be sequenced using well known techniques employing such enzymes as Klenow fragment of DNA polymerase I, Sequenase® (US Biochemical Corp., Cleveland OH), Taq polymerase (Perkin Elmer, Foster City CA), thermostable T7 polymerase (Amersham, Chicago, IL) or

combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System (Gibco BRL, Gaithersburg, MD). An automated sequencing system may be used, using instruments available from commercial suppliers such as Perkin Elmer and Pharmacia.

- 5 The sequence of a partial cDNA may be used to identify a polynucleotide sequence that encodes a full length tumor-associated protein using any of a variety of standard techniques. Within such techniques, a library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, a library is size-selected to include larger molecules.
- 10 Random primed libraries may also be preferred for identifying 5' and upstream regions of genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences.

For hybridization techniques, a partial sequence may be labeled (e.g., by nick-translation or end-labeling with ^{32}P) using well known techniques. A bacterial or bacteriophage library is then screened by hybridizing filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis. cDNA clones may be analyzed to determine the amount of additional sequence by, for example, PCR using a primer from the partial sequence and a primer from the vector. Restriction maps and partial sequences may be generated to identify one or more overlapping clones. The complete sequence may then be determined using standard techniques, which may involve generating a series of deletion clones. The resulting overlapping sequences are then assembled into a single contiguous sequence. A full length cDNA molecule can be generated by ligating suitable fragments, using well known techniques.

Alternatively, there are numerous amplification techniques for obtaining a full length coding sequence from a partial cDNA sequence. Within such techniques, amplification is generally performed via PCR. Any of a variety of commercially available kits may be used to perform the amplification step. Primers may be designed

using, for example, software well known in the art. Primers are preferably 22-30 nucleotides in length, have a GC content of at least 50% and anneal to the target sequence at temperatures of about 68°C to 72°C. The amplified region may be sequenced as described above, and overlapping sequences assembled into a contiguous sequence.

One such amplification technique is inverse PCR (see Triglia et al., *Nucl. Acids Res.* 16:8186, 1988), which uses restriction enzymes to generate a fragment in the known region of the gene. The fragment is then circularized by intramolecular ligation and used as a template for PCR with divergent primers derived from the known region. Within an alternative approach, sequences adjacent to a partial sequence may be retrieved by amplification with a primer to a linker sequence and a primer specific to a known region. The amplified sequences are typically subjected to a second round of amplification with the same linker primer and a second primer specific to the known region. A variation on this procedure, which employs two primers that initiate extension in opposite directions from the known sequence, is described in WO 96/38591. Additional techniques include capture PCR (Lagerstrom et al., *PCR Methods Appl.* 1:111-19, 1991) and walking PCR (Parker et al., *Nucl. Acids. Res.* 19:3055-60, 1991). Other methods employing amplification may also be employed to obtain a full length cDNA sequence.

In certain instances, it is possible to obtain a full length cDNA sequence by analysis of sequences provided in an expressed sequence tag (EST) database, such as that available from GenBank. Searches for overlapping ESTs may generally be performed using well known programs (e.g., NCBI BLAST searches), and such ESTs may be used to generate a contiguous full length sequence.

Nucleic acid sequences of partial cDNA molecules B7 and B11, which encode tumor associated proteins, are provided in Figures 1 and 2 (and SEQ ID NOs:1 and 2), respectively. B7 and B11 were isolated using T-RExCS, and are highly expressed in tumor cells. These sequences are expressed at a lower level, or undetectably, in normal tissues examined by Northern blot analysis. These polynucleotides, as well as full length polynucleotides comprising a recited sequence,

other portions of full length polynucleotides, and sequences complementary to all or a portion of such full length molecules, are specifically encompassed by the present invention.

Variants of the recited polynucleotide sequences are also provided herein. Polynucleotide variants may contain one or more substitutions, deletions, insertions and/or modifications such that the antigenic, immunogenic and/or biological properties of the encoded polypeptide are not diminished. The effect on the properties of the encoded polypeptide may generally be assessed as described herein. Preferred variants contain nucleotide substitutions, deletions, insertions and/or modifications at no more than 20%, preferably at no more than 10%, of the nucleotide positions. Certain variants are substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a tumor-associated protein (or a complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65° C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention.

As noted above, portions of any of the above sequences are also contemplated by the present invention. Such polynucleotides may generally be prepared by any method known in the art, including chemical synthesis by, for example, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding a tumor-associated protein, or a portion thereof, provided that the DNA is incorporated into a

vector with a suitable RNA polymerase promoter (such as T7 or SP6). Certain portions may be used to prepare an encoded polypeptide, as described herein. In addition, or alternatively, a portion may function as a probe (e.g., for diagnostic purposes), and may be labeled by a variety of reporter groups, such as radionuclides and enzymes. Such portions are preferably at least 10 nucleotides in length, more preferably at least 20 nucleotides in length and still more preferably at least 30 nucleotides in length.

A portion of a sequence complementary to a coding sequence (*i.e.*, an antisense polynucleotide) may also be used as a probe or to modulate gene expression. cDNA constructs that can be transcribed into antisense RNA may also be introduced into cells of tissues to facilitate the production of antisense RNA. An antisense polynucleotide may be used, as described herein, to inhibit expression of a tumor-associated gene. Antisense technology can be used to control gene expression through triple-helix formation, which compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors or regulatory molecules (see Gee et al., *In Huber and Carr, Molecular and Immunologic Approaches*, Futura Publishing Co. (Mt. Kisco, NY; 1994). Alternatively, an antisense molecule may be designed to hybridize with a control region of a gene (*e.g.*, promoter, enhancer or transcription initiation site), and block transcription of the gene; or to block translation by inhibiting binding of a transcript to ribosomes.

Any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2'-O-methyl rather than phosphodiester linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl-, methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Nucleotide sequences as described herein may be joined to a variety of other nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation

vectors and sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, convenient restriction endonuclease sites and one or more selectable markers. Other elements will depend upon the desired use, and will be apparent to those of ordinary skill in the art.

5 Within certain embodiments, polynucleotides may be formulated so as to permit entry into a cell of a mammal, and expression therein. Such formulations are particularly useful for therapeutic purposes, as described below. Those of ordinary skill in the art will appreciate that there are many ways to achieve expression of a polynucleotide in a target cell, and any suitable method may be employed. For
10 example, a polynucleotide may be incorporated into a viral vector such as, but not limited to, adenovirus, adeno-associated virus, retrovirus, or vaccinia or other pox virus (e.g., avian pox virus). Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or selection of
15 transduced cells) and/or a targeting moiety, such as a gene that encodes a ligand for a receptor on a specific target cell, to render the vector target specific. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art. cDNA constructs within such a vector may be used, for example, to transfet human or animal cell lines for use in establishing tumor models which may be used to
20 perform tumor protection and adoptive immunotherapy experiments to demonstrate tumor or leukemia-growth inhibition or lysis of such cells.

Other therapeutic formulations for polynucleotides include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed
25 micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (i.e., an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

TUMOR-ASSOCIATED POLYPEPTIDES

Polypeptides within the scope of the present invention comprise at least a portion of a tumor-associated protein or variant thereof, where the portion is immunologically and/or biologically active. Such polypeptides may be of any length, 5 including a full length protein, an oligopeptide (*i.e.*, consisting of a relatively small number of amino acid residues, such as 8-10 residues, joined by peptide bonds), or a peptide of intermediate length. Polypeptides comprising relatively small portions of a native tumor-associated protein (*e.g.*, less than 23, preferably less than 19 and more preferably less than 16 consecutive amino acid residues) are particularly preferred for 10 use in generating a T cell response. A polypeptide may further comprise additional sequences, which may or may not be derived from a native tumor-associated protein. Such sequences may (but need not) possess immunogenic properties and/or a biological activity.

A polypeptide is "immunologically active," within the context of the 15 present invention if it is recognized (*i.e.*, specifically bound) by a B-cell and/or T-cell surface antigen receptor. Immunological activity may generally be assessed using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides derived from the native polypeptide for the ability to react with 20 antigen-specific antisera and/or T-cell lines or clones, which may be prepared using well known techniques. An immunologically active portion of a tumor-associated protein reacts with such antisera and/or T-cells at a level that is not substantially lower than the reactivity of the full length polypeptide (*e.g.*, in an ELISA and/or T-cell reactivity assay). Such screens may generally be performed using methods well known 25 to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. B-cell and T-cell epitopes may also be predicted via computer analysis.

Alternatively, immunogenic portions may be identified using computer analysis, such as the Tsites program (*see* Rothbard and Taylor, *EMBO J.* 7:93-100, 30 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide

motifs that have the potential to elicit Th responses. CTL peptides with motifs appropriate for binding to murine and human class I or class II MHC may be identified according to BIMAS (Parker et al., *J. Immunol.* 152:163, 1994) and other HLA peptide binding prediction analyses. To confirm immunogenicity, a peptide may be tested 5 using an HLA A2 transgenic mouse model and/or an *in vitro* stimulation assay using dendritic cells, fibroblasts or peripheral blood cells.

Similarly, a polypeptide is "biologically active" if it possesses one or more structural, regulatory and/or biochemical functions of the native tumor-associated protein. A biological activity may be assessed using well known methods. For 10 example, sequence comparisons may indicate a particular biological activity for the protein. Appropriate assays designed to evaluate the activity may then be designed based on existing assays known in the art.

As noted above, polypeptides may comprise one or more portions of a variant of an endogenous protein, where the portion is immunologically and/or 15 biologically active (*i.e.*, the portion exhibits one or more antigenic, immunogenic and/or biological properties characteristic of the full length protein). Preferably, such a portion is at least as active as the full length protein within one or more assays to detect such properties. A polypeptide "variant," as used herein, is a polypeptide that differs from a native protein in substitutions, insertions, deletions and/or amino acid 20 modifications, such that the antigenic, immunogenic and/or biological properties of the native protein are not substantially diminished. A variant preferably retains at least 80% sequence identity to a native sequence, more preferably at least 90% identity, and even more preferably at least 95% identity. Guidance in determining which and how many amino acid residues may be substituted, inserted, deleted and/or modified without 25 diminishing immunological and/or biological activity may be found using any of a variety of computer programs known in the art, such as DNAStar software. Properties of a variant may generally be evaluated by assaying the reactivity of the variant with antisera and/or T-cells as described above and/or evaluating a biological property characteristic of the native protein.

Certain variants contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

Variants within the scope of this invention also include polypeptides in which the primary amino acid structure of a native protein is modified by forming covalent or aggregative conjugates with other polypeptides or chemical moieties such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives may be prepared, for example, by linking particular functional groups to amino acid side chains or at the N- or C-termini.

The present invention also includes polypeptides with or without associated native-pattern glycosylation. Polypeptides expressed in yeast or mammalian expression systems may be similar to or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system.

Expression of DNA in bacteria such as *E. coli* provides non-glycosylated molecules. N-glycosylation sites of eukaryotic proteins are characterized by the amino acid triplet Asn-A₁-Z, where A₁ is any amino acid except Pro, and Z is Ser or Thr. Variants having

inactivated N-glycosylation sites can be produced by techniques known to those of ordinary skill in the art, such as oligonucleotide synthesis and ligation or site-specific mutagenesis techniques, and are within the scope of this invention. Alternatively, N-linked glycosylation sites can be added to a polypeptide.

As noted above, polypeptides may comprise sequences that are not related to an endogenous tumor-associated protein. For example, an N-terminal signal (or leader) sequence may be present, which co-translationally or post-translationally directs transfer of the polypeptide from its site of synthesis to a site inside or outside of the cell membrane or wall. The polypeptide may also comprise a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His or hemagglutinin), or to enhance binding of the polypeptide to a solid support. Fusion proteins capped with such peptides may also be resistant to intracellular degradation in *E. coli*. Protein fusions encompassed by this invention further include, for example, polypeptides conjugated to an immunoglobulin Fc region. All of the above protein fusions may be prepared by chemical linkage or as fusion proteins, as described below.

Also included within the present invention are alleles of a tumor-associated protein. Alleles are alternative forms of a native protein resulting from one or more genetic mutations (which may be amino acid deletions, additions and/or substitutions), resulting in an altered mRNA. Allelic proteins may differ in sequence, but overall structure and function are substantially similar.

Tumor-associated polypeptides, variants and portions thereof may generally be prepared from nucleic acid encoding the desired polypeptide using well known techniques. To prepare an endogenous protein, an isolated cDNA may be used. To prepare a variant polypeptide, standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis may be used, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been

transformed or transfected with an expression vector containing a DNA sequence that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. Following expression, supernatants from 5 host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. One or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide.

10 Certain portions and other variants may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, portions and other variants having fewer than about 500 amino acids, preferably fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be synthesized. Polypeptides may be synthesized using any of the commercially available 15 solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. See Merrifield, J. Am. Chem. Soc. 85:2149-2146, 1963. Various modified solid phase techniques are also available (e.g., the method of Roberge et al., Science 269:202-204, 1995). Equipment for automated synthesis of polypeptides is commercially available from suppliers such 20 as Applied BioSystems, Inc. (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptides and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is 25 separated from some or all of the coexisting materials in the natural system. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

ANTIBODIES AND FRAGMENTS THEREOF

The present invention further provides binding agents, such as antibodies and antigen-binding fragments thereof, that specifically bind to a tumor-associated protein. Within the context of the present invention, an antibody or antigen-binding fragment is said to "specifically bind" to a tumor-associated protein if it reacts at a detectable level (within, for example, an ELISA) with a tumor-associated protein or a portion or variant thereof, and does not react detectably with unrelated proteins. As used herein, "binding" refers to a noncovalent association between two separate molecules, such that a complex is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind," when the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant maybe determined using methods well known in the art.

- Any agent that satisfies the above requirements may be a binding agent.
- In a preferred embodiment, a binding agent is an antibody or antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, such as techniques for generating monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, which allows for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (e.g., mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification.
- Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies

specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane,

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988) and digested by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns.

Monoclonal antibodies and fragments thereof may be coupled to one or 5 more therapeutic or diagnostic agents. Representative therapeutic agents include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. For diagnostic purposes, coupling of radioactive agents may be used to facilitate tracing of metastases or to determine the location of tumors.

A therapeutic or diagnostic agent may be coupled (e.g., covalently bonded) to a suitable monoclonal antibody either directly or indirectly (e.g., via a linker group). A direct reaction between an agent and an antibody is possible when each 10 possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulphydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl 15 group containing a good leaving group (e.g., a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A 20 linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional, may be employed as the 25 linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulphydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology within the patent and scientific literature.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a 30 linker group which is cleavable during or upon internalization into a cell. A number of

different cleavable linker groups have been described within the patent and scientific literature, including groups comprising a disulfide bond, a photolabile bond or a hydrolyzable bond.

It may be desirable to couple multiple agents (of the same type or of different types) to an antibody. Immunoconjugates with multiple agents may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment can be used. Alternatively, a carrier can be used. Certain carriers bear the agents via covalent bonding (directly or via a linker group). Such carriers include proteins such as albumins (e.g., U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (e.g., U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds that contain, for example, nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide.

A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

Also provided herein are anti-idiotypic antibodies that mimic an immunogenic portion of a native protein. Such antibodies may be raised against an antibody, or antigen-binding fragment thereof, that specifically binds to an immunogenic portion of a tumor-associated protein, using well known techniques. Anti-idiotypic antibodies that mimic an immunogenic portion are those antibodies that bind to an antibody, or antigen-binding fragment thereof, that specifically binds to an immunogenic portion of a tumor-associated protein, as described herein.

T CELLS

Immunotherapeutic compositions may also, or alternatively, comprise T cells specific for a tumor-associated protein. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be present within 5 (or isolated from) bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a mammal, such as a patient, using a commercially available cell separation system, such as the CEPRATE™ system, available from CellPro Inc., Bothell WA (see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived 10 from related or unrelated humans, non-human animals, cell lines or cultures.

T cells may be stimulated with a tumor-associated polypeptide, polynucleotide and/or an antigen presenting cell (APC) that expresses such a polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide. Preferably, a 15 polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of antigen-specific T cells. Briefly, T cells, which may be isolated by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes), are incubated with polypeptide. For example, T cells may be incubated *in vitro* for 2-9 days (typically 4 20 days) at 37°C with polypeptide (e.g., 5 to 25 µg/ml) or cells synthesizing a comparable amount of polypeptide. It may be desirable to incubate a separate aliquot of a T cell sample in the absence of polypeptide to serve as a control.

T cells are considered to be specific for a polypeptide if the T cells kill target cells coated with the polypeptide or expressing a gene encoding such a 25 polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res.* 54:1065-1070, 1994. Alternatively, 30 detection of the proliferation of T cells may be accomplished by a variety of known

techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (e.g., by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Other ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca^{2+} flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium. Alternatively, synthesis of lymphokines (such as interferon-gamma) can be measured or the relative number of T cells that can respond to a polypeptide may be quantified. Contact with a polypeptide (200 ng/ml - 100 $\mu\text{g}/\text{ml}$, preferably 100 ng/ml - 25 $\mu\text{g}/\text{ml}$) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells and/or contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (e.g., TNF or IFN- γ) is indicative of T cell activation (see Coligan et al., Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998)). T cells with the desired specificity may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient or a related or unrelated donor and are administered to the patient following stimulation and expansion.

T cells that have been activated in response to a tumor-associated polypeptide, polynucleotide or polypeptide-expressing APC may be CD4 $^{+}$ and/or CD8 $^{+}$. Specific activation of CD4 $^{+}$ or CD8 $^{+}$ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the production of cytokines (e.g., lymphokines), or the generation of cytolytic activity (i.e., generation of cytotoxic T cells specific for the polypeptide). For CD4 $^{+}$ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For CD8 $^{+}$ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

For therapeutic purposes, CD4 $^{+}$ or CD8 $^{+}$ T cells that proliferate in response to the polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to polypeptide, with or

without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a tumor-associated polypeptide. The addition of stimulator cells is preferred where generating CD8⁺ T cell responses. T cells can be grown to large numbers *in vitro* with retention of specificity in response to intermittent restimulation

5 with polypeptide. Briefly, for the primary *in vitro* stimulation (IVS), large numbers of lymphocytes (e.g., greater than 4×10^7) may be placed in flasks with media containing human serum. Polypeptide (e.g., peptide at 10 µg/ml) may be added directly, along with tetanus toxoid (e.g., 5 µg/ml). The flasks may then be incubated (e.g., 37°C for 7 days). For a second IVS, T cells are then harvested and placed in new flasks with

10 2-3 $\times 10^7$ irradiated peripheral blood mononuclear cells. Polypeptide (e.g., 10 µg/ml) is added directly. The flasks are incubated at 37°C for 7 days. On day 2 and day 4 after the second IVS, 2-5 units of interleukin-2 (IL-2) may be added. For a third IVS, the T cells may be placed in wells and stimulated with the individual's own EBV transformed B cells coated with the peptide. IL-2 may be added on days 2 and 4 of each

15 cycle. As soon as the cells are shown to be specific cytotoxic T cells, they may be expanded using a 10 day stimulation cycle with higher IL-2 (20 units) on days 2, 4 and 6.

Alternatively, one or more T cells that proliferate in the presence of polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution. Responder T cells may be purified from the peripheral blood of sensitized patients by density gradient centrifugation and sheep red cell rosetting and established in culture by stimulating with the nominal antigen in the presence of irradiated autologous filler cells. In order to generate CD4⁺ T cell lines, polypeptide is used as the antigenic stimulus and autologous peripheral blood

20 lymphocytes (PBL) or lymphoblastoid cell lines (LCL) immortalized by infection with Epstein Barr virus are used as antigen presenting cells. In order to generate CD8⁺ T cell lines, autologous antigen-presenting cells transfected with an expression vector which produces polypeptide may be used as stimulator cells. Established T cell lines may be cloned 2-4 days following antigen stimulation by plating stimulated T cells at a

25 frequency of 0.5 cells per well in 96-well flat-bottom plates with 1×10^6 irradiated PBL

or LCL cells and recombinant interleukin-2 (rIL2) (50 U/ml). Wells with established clonal growth may be identified at approximately 2-3 weeks after initial plating and restimulated with appropriate antigen in the presence of autologous antigen-presenting cells, then subsequently expanded by the addition of low doses of rIL2 (10 U/ml) 2-3 days following antigen stimulation. T cell clones may be maintained in 24-well plates by periodic restimulation with antigen and rIL2 approximately every two weeks.

Within certain embodiments, allogeneic T-cells may be primed (*i.e.*, sensitized to a polypeptide) *in vivo* and/or *in vitro*. Such priming may be achieved by contacting T cells with a tumor-associated polypeptide, a polynucleotide encoding such a polypeptide or a cell producing such a polypeptide under conditions and for a time sufficient to permit the priming of T cells. In general, T cells are considered to be primed if, for example, contact with a polypeptide results in proliferation and/or activation of the T cells, as measured by standard proliferation, chromium release and/or cytokine release assays as described herein. A stimulation index of more than two fold increase in proliferation or lysis, and more than three fold increase in the level of cytokine, compared to negative controls, indicates T-cell specificity.

PHARMACEUTICAL COMPOSITIONS AND VACCINES

Within certain aspects, compounds such as polypeptides, antibodies, nucleic acid molecules and/or T cells may be incorporated into pharmaceutical compositions or vaccines. Alternatively, a pharmaceutical composition may comprise an antigen-presenting cell transfected with a tumor-associated polypeptide such that the antigen-presenting cell expresses the polypeptide. Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier or excipient. Certain vaccines may comprise one or more polypeptides and a nonspecific immune response enhancer, such as an adjuvant or a liposome (into which the compound is incorporated). Pharmaceutical compositions and vaccines may additionally contain a delivery system, such as biodegradable microspheres which are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109. Pharmaceutical

compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive.

Within certain embodiments, pharmaceutical compositions and vaccines are designed to elicit T cell responses specific for a tumor-associated polypeptide in a patient, such as a human. In general, T cell responses may be favored through the use of relatively short polypeptides (e.g., comprising less than 23 consecutive amino acid residues of a native polypeptide, preferably 4-16 consecutive residues, more preferably 8-16 consecutive residues and still more preferably 8-10 consecutive residues). Alternatively, or in addition, a vaccine may comprise a non-specific immune response enhancer that preferentially enhances a T cell response. In other words, the immune response enhancer may enhance the level of a T cell response to a polypeptide by an amount that is proportionally greater than the amount by which an antibody response is enhanced. For example, when compared to a standard oil based adjuvant, such as CFA, an immune response enhancer that preferentially enhances a T cell response may enhance a proliferative T cell response by at least two fold, a lytic response by at least 10%, and/or T cell activation by at least two fold compared to negative control cell lines, while not detectably enhancing an antibody response. The amount by which a T cell or antibody response to a polypeptide is enhanced may generally be determined using any representative technique known in the art, such as the techniques provided herein.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. The DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerin*) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (e.g., vaccinia or other pox virus,

retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science* 259:1745-1749, 1993 and reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

As noted above, a pharmaceutical composition or vaccine may comprise an antigen-presenting cell that expresses a tumor-associated polypeptide. For therapeutic purposes, as described herein, the antigen presenting cell is preferably an autologous dendritic cell. Such cells may be prepared and transfected using standard techniques, such as those described by Reeves et al., *Cancer Res.* 56:5672-5677, 1996; Tuting et al., *J. Immunol.* 160:1139-1147, 1998; and Nair et al., *Nature Biotechnol.* 16:364-369, 1998). Expression of a polypeptide on the surface of an antigen-presenting cell may be confirmed by *in vitro* stimulation and standard proliferation as well as chromium release assays, as described herein.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. For certain topical applications, formulation as a cream or lotion, using well known components, is preferred.

Such compositions may also comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextran), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of adjuvants may be employed in the vaccines of this invention to nonspecifically enhance the immune response. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable non-specific immune response enhancers include alum-based adjuvants (e.g., Alhydrogel, Rehydragel, aluminum phosphate, Algammulin, aluminum hydroxide); oil based adjuvants (e.g., Freund's adjuvant, Specol, RIBI, TiterMax, Montanide ISA50 or Seppic MONTANIDE ISA 720); cytokines (e.g., GM-CSF, interleukin-2, -7, or -12, or Flat3-ligand); microspheres; nonionic block copolymer-based adjuvants; dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants AS-1, AS-2 (Smith Kline Beecham); Ribi Adjuvant system based adjuvants; QS21 (Aquila); saponin based adjuvants (crude saponin, the saponin Quil A); muramyl dipeptide (MDP) based adjuvants such as SAF (Syntex adjuvant in its microfluidized form (SAF-m)); dimethyl-dioctadecyl ammonium bromide (DDA); human complement based adjuvants *m. vaccae* and derivatives; immune stimulating complex (iscom) based adjuvants; inactivated toxins; and attenuated infectious agents (such as *M. tuberculosis*).

As noted above, within certain embodiments, immune response enhancers may be chosen for their ability to preferentially elicit or enhance a T cell response (e.g., CD4⁺ and/or CD8⁺) to a tumor-associated polypeptide. Such immune response enhancers are well known in the art, and include (but are not limited to) Montanide ISA50, Seppic MONTANIDE ISA 720, cytokines (e.g., GM-CSF, Flat3-ligand), microspheres, dimethyl dioctadecyl ammoniumbromide (DDA) based

adjuvants, AS-1 (Smith Kline Beecham), AS-2 (Smith Kline Beecham), Ribi Adjuvant system based adjuvants, QS21 (Aquila), saponin based adjuvants (crude saponin, the saponin Quil A), Syntex adjuvant in its microfluidized form (SAF-m), MV, ddMV (Genesis), immune stimulating complex (iscom) based adjuvants and inactivated toxins.

- 5 The compositions described herein may be administered as part of a sustained release formulation (*i.e.*, a formulation such as a capsule or sponge that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site.
- 10 Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. The amount of active compound contained
- 15 within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

CANCER THERAPY

- 20 In further aspects of the present invention, the compositions and vaccines described herein may be used for therapy of cancer such as breast cancer. Suitable patients for therapy may be any warm-blooded animal, preferably a human. A patient may or may not be afflicted with cancer, as determined by standard diagnostic methods. Accordingly, the above pharmaceutical compositions and vaccines may be used to
- 25 prevent the development of cancer or to treat a patient afflicted with cancer. In addition, the compositions provided herein may be used alone or in combination with conventional therapeutic regimens such as surgery, irradiation, chemotherapy and/or bone marrow transplantation.

30 Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The

route, duration and frequency of administration will be determined by such factors as the condition of the patient, the type and severity of the patient's disease and the method of administration. Routes and frequency of administration may vary from individual to individual, and may be readily established using standard techniques. In general, the 5 pharmaceutical compositions and vaccines may be administered by injection (e.g., intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (e.g., by aspiration) or orally. Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be 10 appropriate for individual patients.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. For example, certain pharmaceutical compositions and vaccines may be administered in an amount capable of promoting an anti-tumor immune response. Such 15 a response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Treatment with a pharmaceutical composition or vaccine should also lead to an improved clinical outcome (e.g., more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to untreated 20 patients.

Within particularly preferred embodiments of the invention, a polypeptide may be administered at a dosage ranging from about 100 µg to 5 mg per kg of host, on a regimen of single or multiple daily doses. DNA molecules encoding such polypeptides may generally be administered in amounts sufficient to generate 25 comparable levels of polypeptide. Appropriate dosages of polypeptides, polynucleotides and antibodies may generally be determined using experimental models and/or clinical trials. In general, the use of the minimum dosage that is sufficient to provide effective therapy is preferred. Patients may generally be monitored for therapeutic effectiveness using assays suitable for the condition being treated or 30 prevented, which will be familiar to those of ordinary skill in the art. Suitable dose

sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

Within other embodiments, an antisense polynucleotide may be administered to inhibit expression of a tumor-associated gene. Antisense technology can be used to control gene expression through triple-helix formation, which compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors or regulatory molecules (see Gee et al., *In Huber and Carr, Molecular and Immunologic Approaches*, Futura Publishing Co. (Mt. Kisco, NY; 1994). Alternatively, an antisense molecule may be designed to hybridize with a control region of a gene (e.g., promoter, enhancer or transcription initiation site), and block transcription of the gene; or to block translation by inhibiting binding of a transcript to ribosomes.

Within further aspects, methods for inhibiting the development of a cancer involve the administration of autologous T cells that have been activated in response to a tumor-associated polypeptide or APC. Such T cells may be CD4⁺ and/or CD8⁺, and may be proliferated as described above. The T cells may be administered to the individual in an amount effective to inhibit the development of a malignant disease. Typically, about 1×10^9 to 1×10^{11} T cells/M² are administered intravenously, intracavitory or in the bed of a resected tumor. It will be evident to those skilled in the art that the number of cells and the frequency of administration will be dependent upon the response of the patient.

CANCER DETECTION, DIAGNOSIS AND MONITORING

Polypeptides, polynucleotides and antibodies, as described herein, may be used within a variety of methods for detecting a cancer in a patient. Without wishing to be bound to any specific theory, it is believed that the presence tumor-associated polypeptides and/or polynucleotides described herein in the cells of a host is indicative of the presence of cancer. Further, the presence of such polypeptides and/or polynucleotides in non-tumor tissue is believed to be indicative of metastasis.

Diagnostic methods may also be based on the detection of a immune response specific for the tumor-associated protein (cellular or humoral) in a patient.

Methods involving the use of an antibody may detect the presence or absence of a polypeptide as described herein in any suitable biological sample. Suitable biological samples include tumor or normal tissue biopsy, mastectomy, blood, lymph node, serum or urine samples, or other tissue, homogenate or extract thereof obtained from a patient.

There are a variety of assay formats known to those of ordinary skill in the art for using an antibody to detect polypeptide markers in a sample. See, e.g., 10 Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. For example, the assay may be performed in a Western blot format, wherein a protein preparation from the biological sample is submitted to gel electrophoresis, transferred to a suitable membrane and allowed to react with the antibody. The presence of the antibody on the membrane may then be detected using a suitable 15 detection reagent, as described below.

In another embodiment, the assay involves the use of antibody immobilized on a solid support to bind to the polypeptide and remove it from the remainder of the sample. The bound polypeptide may then be detected using a second antibody or reagent that contains a reporter group. Alternatively, a competitive assay 20 may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized antibody after incubation of the antibody with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the antibody is indicative of the reactivity of the sample with the immobilized antibody, and as a result, indicative of the concentration of polypeptide in the sample.

25 The solid support may be any material known to those of ordinary skill in the art to which the antibody may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose filter or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a

magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681.

The antibody may be immobilized on the solid support using a variety of techniques known to those in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, 10 adsorption may be achieved by contacting the antibody, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of antibody ranging from about 10 ng to about 1 μ g, and preferably about 100-200 ng, is 15 sufficient to immobilize an adequate amount of polypeptide.

Covalent attachment of antibody to a solid support may also generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the antibody. For example, the antibody may be covalently attached to supports having an 20 appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner using well known techniques.

In certain embodiments, the assay for detection of polypeptide in a sample is a two-antibody sandwich assay. This assay may be performed by first 25 contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the biological sample, such that the polypeptide within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a second antibody (containing a reporter group) capable of binding to a different site on the polypeptide is

added. The amount of second antibody that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically 5 blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20TM (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact 10 time (*i.e.*, incubation time) is that period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary 15 skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20TM. The second antibody, which contains a reporter group, may then be added to the solid support. 20 Preferred reporter groups include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. The conjugation of antibody to reporter group may be achieved using standard methods known to those of ordinary skill in the art.

The second antibody is then incubated with the immobilized antibody- 25 polypeptide complex for an amount of time sufficient to detect the bound polypeptide.

An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound second antibody is then removed and bound second antibody is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For 30 radioactive groups, scintillation counting or autoradiographic methods are generally

appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value established from non-tumor tissue. In one preferred embodiment, the cut-off value is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value may be considered positive for cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, p. 106-7 (Little Brown and Co., 1985). Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the antibody is immobilized on a membrane, such as nitrocellulose. In the flow-through test, the polypeptide within the sample bind to the immobilized antibody as the sample passes through the membrane. A second, labeled

- antibody then binds to the antibody-polypeptide complex as a solution containing the second antibody flows through the membrane. The detection of bound second antibody may then be performed as described above. In the strip test format, one end of the membrane to which antibody is bound is immersed in a solution containing the sample.
- 5 The sample migrates along the membrane through a region containing second antibody and to the area of immobilized antibody. Concentration of second antibody at the area of immobilized antibody indicates the presence of cancer. Typically, the concentration of second antibody at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the
- 10 amount of antibody immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 µg, and more preferably from about 50
- 15 ng to about 1 µg. Such tests can typically be performed with a very small amount of biological sample.
- The presence or absence of a cancer in a patient may also be determined by evaluating the level of mRNA encoding a polypeptide of the present invention within the biological sample (e.g., a biopsy, mastectomy and/or blood sample from a
- 20 patient) relative to a predetermined cut-off value. Such an evaluation may be achieved using any of a variety of methods known to those of ordinary skill in the art such as, for example, *in situ* hybridization and amplification by polymerase chain reaction. Probes and primers for use within such assays may generally be designed based on the sequences recited herein, or on similar sequences identified in other individuals. Probes
- 25 may be used within well known hybridization techniques, and may be labeled with a detection reagent to facilitate detection of the probe. Such reagents include, but are not limited to, radionuclides, fluorescent dyes and enzymes capable of catalyzing the formation of a detectable product.

Primers may generally be used within detection methods involving

30 polymerase chain reaction (PCR), such as RT-PCR, in which PCR is applied in

conjunction with reverse transcription. Typically, RNA is extracted from a sample tissue and is reverse transcribed to produce cDNA molecules. PCR amplification using specific primers generates a tumor-associated cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification is typically 5 performed on samples obtained from matched pairs of tissue (tumor and non-tumor tissue from the same individual) or from unmatched pairs of tissue (tumor and non-tumor tissue from different individuals). The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the tumor sample as compared to the same 10 dilutions of the non-tumor sample is typically considered positive.

In yet another method for determining the presence or absence of a cancer in a patient, one or more of the polypeptides described above may be used in a skin test. As used herein, a "skin test" is any assay performed directly on a patient in which a delayed-type hypersensitivity (DTH) reaction (such as swelling, reddening or 15 dermatitis) is measured following intradermal injection of one or more polypeptides as described above. Such injection may be achieved using any suitable device sufficient to contact the polypeptide or polypeptides with dermal cells of the patient, such as a tuberculin syringe or 1 mL syringe. Preferably, the reaction is measured at least 48 hours after injection, more preferably 48-72 hours.

20 The DTH reaction is a cell-mediated immune response, which is greater in patients that have been exposed previously to a test antigen (*i.e.*, an immunogenic portion of a polypeptide employed, or a variant thereof). The response may be measured visually, using a ruler. In general, a response that is greater than about 0.5 cm in diameter, preferably greater than about 5.0 cm in diameter, is a positive response, 25 indicative of a cancer.

The polypeptides of this invention are preferably formulated, for use in a skin test, as pharmaceutical compositions containing at least one polypeptide and a physiologically acceptable carrier, such as water, saline, alcohol, or a buffer. Such compositions typically contain one or more of the above polypeptides in an amount 30 ranging from about 1 μ g to 100 μ g, preferably from about 10 μ g to 50 μ g in a volume

of 0.1 mL. Preferably, the carrier employed in such pharmaceutical compositions is a saline solution with appropriate preservatives, such as phenol and/or Tween 80™.

Certain *in vivo* diagnostic assays may be performed directly on the tumor. One such assay involves contacting tumor cells with an antibody or fragment thereof that binds to a tumor-associated protein. The bound antibody or fragment may then be detected directly or indirectly via a reporter group. Such antibodies may also be used in histological applications.

As noted above, the level of an immune response specific for a tumor-associated protein may be used to determine the presence or absence of a cancer. For example, a patient may be tested for the level of T cells specific for a tumor-associated protein. Within certain methods, a biological sample comprising CD4⁺ and/or CD8⁺ T cells isolated from a patient is incubated with a tumor-associated polypeptide, a polynucleotide encoding a tumor-associated polypeptide and/or an APC that expresses a tumor-associated polypeptide, and the presence or absence of specific activation of the T cells is detected, as described herein. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with a tumor-associated polypeptide (e.g., 5 - 25 µg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of tumor-associated polypeptide to serve as a control. For CD4⁺ T cells, activation is preferably detected by evaluating proliferation of the T cells. For CD8⁺ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer. Further correlation may be made, using methods well known in the art, between the level of proliferation and/or cytolytic activity and the predicted response to therapy. In particular, patients that display a higher antibody, proliferative and/or lytic response may be expected to show a greater response to therapy.

Within other methods, a biological sample obtained from a patient is tested for the level of antibody specific for a tumor-associated polypeptide. The biological sample is incubated with a tumor-associated polypeptide, a polynucleotide encoding a tumor-associated polypeptide and/or an APC that expresses a tumor-associated polypeptide under conditions and for a time sufficient to allow immunocomplexes to form. Immunocomplexes formed between the a tumor-associated polypeptide and antibodies in the biological sample that specifically bind to the tumor-associated polypeptide are then detected. A biological sample for use within such methods may be any sample obtained from a patient that would be expected to contain antibodies. Suitable biological samples include blood, sera, ascites, bone marrow, pleural effusion, and cerebrospinal fluid.

The biological sample is incubated with the tumor-associated polypeptide in a reaction mixture under conditions and for a time sufficient to permit immunocomplexes to form between the polypeptide and antibodies specific for the tumor-associated polypeptide. For example, a biological sample and tumor-associated polypeptide may be incubated at 4°C for 24-48 hours.

Following the incubation, the reaction mixture is tested for the presence of immunocomplexes. Detection of immunocomplexes formed between the tumor-associated polypeptide and antibodies present in the biological sample may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISA). Suitable assays are well known in the art and are amply described in the scientific and patent literature (e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988). Assays that may be used include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Patent 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide et al., in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Patent 4,452,901); immunoprecipitation of labeled ligand (Brown et al., *J. Biol. Chem.* 255:4980-4983, 1980); enzyme-linked immunosorbent assays as described by, for example, Raines and Ross, *J. Biol. Chem.*

257,5154-5160, 1982; immunocytochemical techniques, including the use of fluorochromes (Brooks et al., *Clin. Exp. Immunol.* 39: 477, 1980); and neutralization of activity (Bowen-Pope et al., *Proc. Natl. Acad. Sci. USA* 81:2396-2400, 1984). Other immunoassays include, but are not limited to, those described in U.S. Patent Nos.: 5 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

For detection purposes, a tumor-associated polypeptide may either be labeled or unlabeled. Unlabeled tumor-associated polypeptide may be used in agglutination assays or in combination with labeled detection reagents that bind to the 10 immunocomplexes (e.g., anti-immunoglobulin, protein G, protein A or a lectin and secondary antibodies, or antigen-binding fragments thereof, capable of binding to the antibodies that specifically bind to the tumor-associated polypeptide). If the tumor-associated polypeptide is labeled, the reporter group may be any suitable reporter group known in the art, including radioisotopes, fluorescent groups, luminescent groups, 15 enzymes, biotin and dye particles.

Within certain assays, unlabeled tumor-associated polypeptide is immobilized on a solid support. The solid support may be any material known to those of ordinary skill in the art to which the polypeptide may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable 20 membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The polypeptide may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply 25 described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is 30 preferred. In such cases, adsorption may be achieved by contacting the tumor-

associated polypeptide; in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of polypeptide ranging from about 5 10 ng to about 10 µg, and preferably about 100 ng to about 1 µg, is sufficient to immobilize an adequate amount of polypeptide.

Following immobilization, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin, Tween 20TM (Sigma Chemical Co., St. 10 Louis, MO), heat-inactivated normal goat serum (NGS), or BLOTTTO (buffered solution of nonfat dry milk which also contains a preservative, salts, and an antifoaming agent). The support is then incubated with a biological sample suspected of containing specific antibody. The sample can be applied neat, or, more often, it can be diluted, usually in a buffered solution which contains a small amount (0.1%-5.0% by weight) of protein, 15 such as BSA, NGS, or BLOTTTO. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of antibody that specifically binds the tumor-associated polypeptide within a sample containing such an antibody. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound 20 antibody. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support 25 with an appropriate buffer, such as PBS containing 0.1% Tween 20TM. A detection reagent that binds to the immunocomplexes and that comprises a reporter group may then be added. The detection reagent is incubated with the immunocomplex for an amount of time sufficient to detect the bound antibody. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period 30 of time. Unbound detection reagent is then removed and bound detection reagent is

- detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups.
- 5 Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase and glucose oxidase) may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.
- 10 Regardless of the specific method employed, a level of bound detection reagent that is at least two fold greater than background (*i.e.*, the level observed for a biological sample obtained from a disease-free individual) indicates the presence of a malignant disease associated with tumor-associated polypeptide expression.
- In other aspects of the present invention, the progression and/or response
- 15 to treatment of a cancer may be monitored by performing any of the above assays over a period of time, and evaluating the change in the amount of polypeptide or mRNA detected, or in the extent of the immune response detected. For example, the assays may be performed every month to every other month for a period of 1 to 2 years. To monitor the effect of a therapy or immunization, assays may be performed before and
- 20 after treatment or immunization. In general, a cancer is progressing in those patients in whom the level of tumor-associated polypeptide increases over time. In contrast, a cancer is not progressing when the level of tumor-associated polypeptide detected either remains constant or decreases with time. For methods in which an immune response is detected, a statistically significant increase in the immune response detected following
- 25 therapy or immunization reflects successful therapy or immunization.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing the assay. Such components may be compounds, reagents and/or containers or equipment. For example, one container within a kit may contain a

30 monoclonal antibody or fragment thereof that specifically binds to a tumor-associated

polypeptide. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also contain a detection reagent (e.g., an antibody) that contains a reporter group suitable for direct or 5 indirect detection of antibody binding.

METHODS FOR IDENTIFYING BINDING AGENTS AND MODULATING AGENTS

The present invention further provides methods for identifying compounds that bind to and/or modulate the activity of a tumor-associated protein:

10 Such agents may generally be identified by contacting a polypeptide as provided herein with a candidate compound or agent under conditions and for a time sufficient to allow interaction with the polypeptide. Any of a variety of well known binding assays may then be performed to assess the ability of the candidate compound to bind to the polypeptide, and assays for a biological activity of the polypeptide may be performed to 15 identify agents that modulate (*i.e.*, enhance or inhibit) the biological activity of the protein. Depending on the design of the assay, a polypeptide may be free in solution, affixed to a solid support, present on a cell surface or located intracellularly.

From the foregoing it will be appreciated that, although specific 20 embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

1. An isolated polypeptide comprising an immunologically active portion of a tumor-associated protein or a variant thereof, wherein the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:1 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:1.
2. An isolated polypeptide comprising an immunologically active portion of a tumor-associated protein or a variant thereof, wherein the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:2 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:2.
3. An isolated polynucleotide comprising at least 10 nucleotides that encode a portion of a tumor-associated protein or a variant thereof, wherein the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:1 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:1.
4. An isolated polynucleotide comprising at least 10 nucleotides that encode a portion of a tumor-associated protein or a variant thereof, wherein the tumor-associated protein comprises an amino acid sequence encoded by (a) a nucleotide sequence recited in SEQ ID NO:2 or (b) a sequence complementary to a nucleotide sequence recited in SEQ ID NO:2.
5. An expression vector comprising a polynucleotide according to claim 3 or claim 4.
6. A host cell transformed or transfected with an expression vector according to claim 5.
7. A pharmaceutical composition, comprising:

- (a) a polypeptide according to claim 1; and
- (b) a physiologically acceptable carrier or excipient.

8. A pharmaceutical composition, comprising:

- (a) a polypeptide according to claim 2; and
- (b) a physiologically acceptable carrier or excipient.

9. A vaccine, comprising:

- (a) a polypeptide according to claim 1; and
- (b) a nonspecific immune response enhancer.

10. A vaccine, comprising:

- (a) a polypeptide according to claim 2; and
- (b) a nonspecific immune response enhancer.

11. A vaccine according to claim 9 or claim 10 wherein the immune response enhancer is an adjuvant.

12. A pharmaceutical composition, comprising:

- (a) a polynucleotide according to claim 3; and
- (b) a physiologically acceptable carrier or excipient.

13. A pharmaceutical composition, comprising:

- (a) a polynucleotide according to claim 4; and
- (b) a physiologically acceptable carrier or excipient.

14. A vaccine, comprising:

- (a) a polynucleotide according to claim 3; and
- (b) a nonspecific immune response enhancer.

15. A vaccine, comprising:

- (a) a polynucleotide according to claim 4; and

(b) a nonspecific immune response enhancer.

16. A monoclonal antibody, or antigen-binding fragment thereof, that specifically binds to a polypeptide according to claim 1 or claim 2.

17. A pharmaceutical composition, comprising:

- (a) an antibody or fragment thereof according to claim 16; and
- (b) a physiologically acceptable carrier or excipient.

18. A pharmaceutical composition, comprising:

- (a) a T cell that specifically reacts with a polypeptide according to claim 1 or claim 2; and
- (b) a physiologically acceptable carrier or excipient.

19. A pharmaceutical composition, comprising:

- (a) an antigen presenting cell that expresses a polypeptide according to claim 1 or claim 2; and
- (b) a physiologically acceptable carrier or excipient.

20. A vaccine, comprising:

- (a) an antigen presenting cell that expresses a polypeptide according to claim 1 or claim 2; and
- (b) a non-specific immune response enhancer.

21. A vaccine comprising:

- (a) an anti-idiotypic antibody or antigen-binding fragment thereof that is specifically bound by an antibody that specifically binds to a polypeptide according to claim 1 or claim 2; and
- (b) non-specific immune response enhancer.

22. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition according to any one of claims 8, 9, 12 or 13.

23. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a vaccine according to any one of claims 10, 11, 14 or 15.

24. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition according to claim 17.

25. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition according to claim 18.

26. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition according to claim 19.

27. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a vaccine according to claim 20.

28. A method for inhibiting the development of breast cancer in a patient, comprising administering to a patient a vaccine according to claim 21.

29. A method for determining the presence or absence of breast cancer in a patient comprising assaying a biological sample obtained from a patient for the presence of a polypeptide according to claim 1 or claim 2, and therefrom determining the presence or absence of breast cancer in the patient.

30. A method according to claim 29 wherein the biological sample is a portion of a tumor.

31. A method according to claim 29 wherein the step of detecting comprises contacting the biological sample with a monoclonal antibody that specifically recognizes a polypeptide according to claim 1 or claim 2.

32. A method for determining the presence or absence of breast cancer in a patient comprising assaying a biological sample obtained from a patient for the presence of a polynucleotide according to claim 3 or claim 4, or a portion thereof, and therefrom determining the presence or absence of breast cancer in the patient.

33. A method according to claim 32 wherein the biological sample is a portion of a tumor.

34. A method according to claim 32 wherein the step of detecting comprises:

- (a) preparing cDNA from RNA molecules in the biological sample; and
- (b) specifically amplifying cDNA molecules that are capable of encoding at least a portion of a polypeptide according to claim 1 or claim 2.

35. A method for monitoring the progression of breast cancer in a patient, comprising:

- (a) detecting, in a biological sample obtained from a patient, an amount of a polypeptide according to claim 1 or claim 2 at a first point in time;
- (b) repeating step (a) at a subsequent point in time; and
- (c) comparing the amounts of polypeptide detected in steps (a) and (b),

and therefrom monitoring the progression of breast cancer in the patient.

36. A method according to claim 35 wherein the biological sample is a portion of a tumor.

37. A method according to claim 35 wherein the step of detecting comprises contacting a portion of the biological sample with a monoclonal antibody that specifically recognizes a polypeptide according to claim 1 or claim 2.

38. A method for monitoring the progression of breast cancer in a patient, comprising:

- (a) detecting, in a biological sample obtained from a patient, an amount of an RNA molecule encoding a polypeptide according to claim 1 or claim 2 at a first point in time;
- (b) repeating step (a) at a subsequent point in time; and
- (c) comparing the amounts of RNA molecules detected in steps (a) and (b), and therefrom monitoring the progression of breast cancer in the patient.

39. A method according to claim 38 wherein the step of detecting comprises:

- (a) preparing cDNA from RNA molecules in the biological sample; and
- (b) specifically amplifying cDNA molecules that are capable of encoding at least a portion of a polypeptide according to claim 1 or claim 2.

40. A diagnostic kit, comprising:

- (a) a monoclonal antibody or fragment thereof according to claim 16; and
- (b) a second monoclonal antibody or fragment thereof that binds to
 - (i) a monoclonal antibody recited in step (a); or
 - (ii) a polypeptide according to claim 1 or claim 2;

wherein the second monoclonal antibody is conjugated to a reporter group.

41. A method for preparing a polypeptide according to claim 1 or claim 2, comprising the steps of:

- (a) culturing a host cell according to claim 6 under conditions suitable for the expression of the polypeptide; and
- (b) recovering the polypeptide from the host cell culture.

1/2

FIG. 1**NOVEL BREAST TUMOR ASSOCIATED SEQUENCE B7**

GATCACGTTCCTTCTGGTGGCGCCACCGTGTGCCGGGCAACCGAAACCCGCC
GATCTTCGGCGGAGTCGGTCAGTAACGGCCACCGTATTGTGGGCTGGGACGGCTCTACATC
ACCCCTGACCAAGGGCGAAGGC GGACCGTGGGATTCCTCTGGCTGGCATGGCTCTCCGCT

2/2

FIG. 2**NOVEL BREAST TUMOR ASSOCIATED SEQUENCE BII**

GGCACGGAGGACTGACCCATCTACTGAACACCCATCTCAAAGCACTAAGCTGGTAGTTGCAAGT
TATAACTGGCCAAAATAAGTAAAATGGAGGGCAGTGTGCAC TGAGTTACTGTTTGGTGA
GATACCTCTAGAGGTGCTGCTCATGTTGGAATTATGTTATGATTCTTGATTGGAGAC
ATTTACTAAACGCACCTAGAACGATTTAAAAGTTGATAGTGACAAAAAGACTACAAGCAA
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ACTAAAAGGCCATGAATAAGAGAGCAAGGAAGGGTATGTTGAGGAGTTGGAGGAAG
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TTTTTTTTTTT

1/1

SEQUENCE LISTING

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BREAST CANCER

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<212> DNA

<213> Homo sapiens

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